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Protons regulate the excitability properties of rat myelinated sensory axons in vitro through block of persistent sodium currents

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Abstract

Little information is available on the pH sensitivity of the excitability properties of mammalian axons. Computer-assisted threshold tracking in humans has helped to define clinically relevant changes of nerve excitability in response to hyperventilation and ischaemia, but *in vivo* studies cannot directly differentiate between the impact of pH and other secondary factors. In this investigation we applied an excitability testing protocol to a rat saphenous skin-nerve preparation *in vitro*. Changes in extracellular pH were induced by altering pCO_2 in the perfusate, and excitability properties of large myelinated fibres were measured in the pH range from 6.9 to 8.1.

The main effect of protons on nerve excitability was a near linear increase in threshold which was accompanied by a decrease in strength-duration time constant reflecting mainly a decrease in persistent sodium current. In the recovery cycle late subexcitability following 7 conditioning stimuli was substantially reduced at acid pH, indicating a block of slow but not of fast potassium channels. Changes in threshold electrotonus were complex, reflecting the combined effects of pH on multiple channel types.

These results provide the first systematic data on pH sensitivity of mammalian nerve excitability properties, and may help in the interpretation of abnormal clinical excitability measurements.

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Introduction

A stable pH at physiological values is a precondition of normal peripheral nerve function. The sensitivity of nerve axons to changes in pH was established in the early 20th century *(Davis et al., 1928; Gasser and Erlanger, 1930; Necheles and Gerard, 1930)*, but our understanding of the mechanisms involved remains incomplete *(Hille, 2001)*. Protons have complex effects on nerve excitability, because they not only have multiple effects on different membrane components, such as ion channels, but these also result in further indirect effects by changing membrane potential and ion concentrations. Protons affect the function of voltage-gated ion channels primarily by altering the surface charge of the axolemma, but also by affecting the permeability of channels to ions . Moreover, protons directly excite some ligand-gated ion channels such as TRPV1 or ASICs (*Leffler et al. 2006*) and thereby alter the excitability of sensory neurons. Furthermore, pH alterations may trigger intracellular ion concentration changes which have important electrophysiological consequences *(Moody, Jr., 1984). In vivo*, changes in pH will result physiologically in changes in potassium levels which in turn have powerful effects on nerve excitability *(Krishnan et al., 2009)*.

Effects of protons can be investigated using threshold tracking, a method of nerve excitability testing which is sensitive to membrane potential, and to changes in membrane potential caused by activation of ion channels and electrogenic ion pumps, including those under the myelin sheath *(Bostock et al., 1998)*. In the past, it has provided evidence that persistent Na^+ currents are very sensitive to H^+ ions and therefore influenced by the acid-base balance *(Mogyoros et al., 1997; Baker and Bostock, 1999)*. This knowledge has helped to explain clinically relevant changes of neuronal excitability in response to rather small extracellular pH shifts *(Baker and Bostock, 1999)*. One clinical study that

compared nerve excitability measurements obtained by threshold-tracking with pH did not show a relationship, but the pH values in the critically ill patients were normal *(Z'graggen et al., 2006)*.

Applying nerve excitability testing to a rodent *in-vitro* model permits the study of pH changes while keeping other factors constant *(Maurer et al., 2007)*. In this preparation we were also able to maintain altered pH levels for several hours, while performing comprehensive excitability testing. We used bicarbonate as a buffer and CO_2 as an uncharged acid which can penetrate cell membranes easily to change pH intracellularly as well as extracellularly *(Vorstrup et al., 1989; Pedersen et al., 1998)*.

Methods and Materials

Animals

Adult female Sprague Dawley rats were used in this study and the results are based on eight experiments in which the full protocol was completed. After killing by cervical dislocation the hair was clipped and the skin of the hind limb with the saphenous nerve attached was removed and mounted corium-side up in an organ bath as described previously *(Reeh, 1986; Kress et al., 1992)*.

Skin nerve in vitro preparation and pH control (Fig. 1)

The corium side was superfused with carbogen-buffered (95% O_2, 5% CO_2) synthetic interstitial fluid (SIF), consisting of 108 mM NaCl, 26. mM NaCO3, 9.6 mM Na+-gluconate, 7.6 mM sucrose, 5.0 mM glucose, 3.5 mM KCl, 1. 7 mM NaH2PO4, 1.5 mM CaCl2 and 0.7 MgSO4, at a pH of 7.40 and a temperature of 32 ℃ *(Bretag, 1969)*. A stainless self-sealing steel ring (inner diameter 12 mm, outer diameter 14 mm, height 10 mm) was placed on top of the nerve to isolate a short segment from the rest of the organ bath. A second stainless steel ring (inner diameter 8 mm, outer diameter 10 mm, height 10 mm) was placed within the first ring without touching it and the fluid between the rings was replaced by liquid paraffin to provide electric isolation from the rest of the organ bath. The inner ring was separately superfused by re-circulating oxygen saturated SIF with a variable flow of CO_2 from a 500 ml reservoir (Fig. 1). The pH was measured continuously with a calibrated pH glass electrode (Hanna pH 210, Hanna Instruments Inc., USA) in the reservoir and could be altered by changing the flow rate of CO_2. There was a 6 minute delay from the change of the pH in the reservoir to the change in the inner ring isolating the saphenous nerve (Fig. 2).

Recordings

The proximal end of the nerve was desheathed and the Aβ compound sensory nerve action potential (SNAP) was recorded (latency to negative peak, amplitude measured peak-to-peak) with gold wire electrodes in a separate recording chamber, which was filled with liquid paraffin, using a commercial isolated amplifier (CED 1902, Cambridge Electronic Design, Cambridge, UK) with filter settings 1Hz to 1kHz. The whole nerve was stimulated approximately 30 mm distally using Ag/AgCl electrodes with uninsulated tip diameters of 2 mm (EPO5, World Precision Instruments, Hertfordshire, UK). The cathode was positioned within the inner ring and the anode 10-30 mm outside the outer ring. The anode was placed in a way to minimize the stimulus artefact and to provide a flat base-line between stimulus artefact and SNAP.

Stimulus protocol (QtracS)

We used a computerized threshold-tracking program (QtracW, ©Institute of Neurology, Queen Square, London, UK; see *(Bostock et al., 1998b)*. The TRONDF recording protocol allowed a comprehensive study of various nerve excitability parameters as well as monitoring selected nerve excitability parameters over time ('Multitrack' function) and is described in more detail in Maurer *et al*., (2007). Stimuli were delivered at a frequency of 1 Hz.

Stimulus-response function. After manually determining the maximal amplitude of the sensory nerve action potential (SNAP) we recorded the stimulus-response function relating stimulus current to SNAP amplitude using 0.5-ms-long test stimuli in steps of 3% of the SNAP maximum. For all subsequent measurements the Qtrac program determined the SNAP amplitude and automatically adjusted the stimulus current in response to different stimulus configurations to evoke a target response of 40 % of the maximal SNAP.

Strength duration relationship. The strength-duration relationship was recorded with decreasing stimulus widths of 0.5 ms, 0.4 ms, 0.2 ms and 0.1 ms. The strength-duration time constant (τ_{SD}) and rheobase was calculated off-line

from thresholds measured according to Weiss's formula *(Weiss, 1901; Bostock, 1983; Mogyoros et al., 1996)*.

Threshold electrotonus. Threshold electrotonus was measured by passing subthreshold depolarizing (+ 40% of the control threshold for 100 ms) or hyperpolarizing (- 40% of the control threshold for 100 ms duration or -120%, of the control threshold for 300ms) currents through the whole nerve. Changes in threshold were tested at defined delays before, during and after the start of the polarizing current ('threshold-electrotonus', *(Bostock and Baker, 1988)*). The control, depolarized and hyperpolarized thresholds were tested in turn. They are graphically represented as threshold reduction to allow visual resemblance of the graphical display in current clamp experiments.

Recovery cycle. The recovery cycle was obtained by measuring the excitability changes following a single or a train of 7 (4 ms inter-stimulus interval) supramaximal (140% of the current required for the maximal SNAP) 1 ms conditioning stimuli, The excitability changes were recorded at 18 conditioning-test intervals, decreasing from 200 ms to 1 ms in an approximately geometric sequence.

Tracking of multiple excitable measures ('Multitrack'). This technique allowed the continuous tracking of several nerve excitability properties over time. We determined (i) strength duration time constant using stimulus durations of 0.1 ms and 0.5 ms, (ii) threshold electrotonus at the end of a 100 ms-depolarizing (+40%) and (iii) threshold electrotonus at the end of a 100 ms-hyperpolarizing (-40%) conditioning stimulus.

Sequence of testing within the experiment. Recordings started using the multitrack function at a neutral (normal) pH of 7.40 (Fig. 2). When parameters were stable for 10 minutes a full nerve excitability test was conducted. Subsequently, we reverted to 'multitracking' and started to change the pH over a period of 20 to 40 minutes. In 4 experiments CO_2 flow was reduced resulting in a progressive shift to basic pH which was allowed to stabilize at 8.10.

The three 'multitrack variables were monitored continuously as the pH was changed , and at the steady state pH of 8.10 a second full nerve excitability test was carried out. The CO_2 flow was then increased and pH lowered to 6.90, while the three 'multitrack' variables were again recorded.

A third set of full nerve excitability measurements was obtained at pH 6.90 before 'multitrack' was resumed and pH normalized to 7.40. In 4 experiments the direction of pH change was reversed from 7.40 to 6.90 to 8.10 and back to 7.40.

Statistics and data analysis. All data are presented as mean ± SEM. Data from comprehensive nerve excitability tests and multitrack recordings were analysed using custom made software package QtracP. Distributions of data points were tested with Lillefors test for normality before group means were compared with a paired *t*-test and repeated measurements tested with ANOVA. $P<0.05$ was considered significant.

Results

In each experiment 3 full nerve excitability tests were conducted. The first recording was always obtained at a neutral baseline pH of 7.40. The second and the third recording were performed when a steady state of the pH has been reached for basic (8.10) or acid (6.90) pH. In half of the experiments pH was altered in the sequence neutral-basic-acid, and in the remaining experiments the order was neutral-acid-basic. There were no significant differences of the results with respect to the direction of pH change. We also monitored selected nerve excitability parameters during the gradual change of pH. The results are presented together with the recordings of the full nerve excitability tests (Table 1). A typical recording of an experiment (Fig.2) shows the occurrence of small threshold changes of the control threshold during the full nerve excitability tests (NET). However, the changes were small compared to those seen during changes in pCO_2. The delay of 6 minutes between the change of the pH (measured in the reservoir, Fig. 2) and the change of the excitability could be attributed to the lag of the pH change at the stimulus site produced by the flow rate and the length of the tubing.

The stimulus response function changes throughout the biological relevant range of pH changes.

The stimulus-response curve relating stimulus current intensity to the maximum amplitude of the SNAP was systematically affected by changes of the pH. pH changes in either direction did not significantly affect the amplitude of the SNAP (pH 6.9: 3.38 ± 0.01 mV; pH 7.4: 3.22 ± 0.01; pH 8.1: 3.42 ± 0.01 mV; P=0.53, ANOVA; Fig. 3 A) or the negative peak latency (pH 6.9: 1.59 ± 0.06 ms; pH 7.4: 1.57 ± 0.04 ms; pH 8.1: 1.57 ± 0.04 ms; P=0.39, ANOVA). However, less current was required to reach a particular response amplitude in basic pH as

compared to acid pH. The current required to reach 50% of the maximal SNAP amplitude was 0.51 ± 0.08 mA at normal pH of 7.40, which was significantly greater than the current required at basic pH (0.39 ± 0.06 mA; P<0.05, paired t-test; Fig. 3 B) and this threshold was higher still at acid pH (0.61 ± 0.10 mA; P<0.05, paired t-test). Over the whole pH range, the threshold current significantly decreased with increasing pH (P<0.01, ANOVA). We found a strong 3rd order polynomial correlation between the control threshold and pH (R=-0.99, P<0.01, Fig. 3C). This function illustrates that small changes in pH affect the excitability threshold of peripheral nerves over the entire biologically relevant range. The most sensitive range is around physiological pH 7.40 where the slope of curve is steepest.

Strength duration time constant increases and rheobase decreases with increasing pH.

Strength-duration time constant (SDTC) increased in parallel to pH (P<0.01, ANOVA). It was significantly longer in basic pH (0.31 ± 0.05 ms) than in normal pH (0.20 ± 0.03 ms, *P*<0.05, paired t-test; Fig. 4A) but not different compared to acid pH (0.20 ± 0.04 ms, P=0.39; Fig. 4B). Monitoring SDTC while continuously changing pH by altering CO_2 concentration revealed that the mean increase in SDTC was best fitted with a 2nd order polynomial fit (R^2=0.99, P<0.01; Fig. 4C).

Rheobase was even more affected by pH. It was lower in basic pH compared to normal pH (pH: 8.1: 0.22 ± 0.12 mA, pH 7.4: 0.30 ± 0.12 mA, P<0.01, paired t-test; Fig. 4B) but not different compared to acid pH (pH 6.9: 0.40 ± 0.12 mA, P=0.38, paired t-test). The overall effect is a decrease of rheobase with increasing pH (P<0.01, ANOVA). Therefore, a basic environment pH seems to affect SDTC and rheobase more (higher sensitivity) than an acid environment.

pH changes affect late subexcitability only after a train of preconditioning supramaximal stimuli.

Recovery cycles were recorded after a single supramaximal conditioning stiumulus and after a volley of 7 supramaximal conditioning stimuli. After one supramaximal conditioning stimulus we did not find any difference in the extent of maximal superexcitability (pH 6.9: -15 ± 2 %; pH 7.4: -16 ± 2 %; pH 8.1: -13 ± 2 %;P=0.38, ANOVA), the extent of the relative refractory period (pH 6.9: 2.5 ± 1.2 ms; pH 7.4: 1.9 ± 1.1 ms; pH 8.1: 2.1 ± 1.2ms; P=0.44, ANOVA) or extent of late subexcitability (pH 6.9: 7 ± 2 %; pH 7.4: 6 ± 1 %; pH 8.1: 7 ± 1 %; P=0.62, ANOVA; Fig. 5A,) measured in different pH solutions. However, changes in pH evoked a significant difference in excitability threshold measured at an interval of 5 ms after 7 conditioning stimuli analysed over the whole pH range (P< 0.01, ANOVA), between normal and acid pH (pH 6.9: 11 ± 5 %; pH 7.4: 27 ± 3 %; P<0.05, paired t-test) and between normal and basic pH (pH 8.1: 39 ± 7 %; P<0.05, paired t-test) (Fig. 5 B). The preconditioning volley of supramaximal stimuli leads *per se* to a disappearance of superexcitability and a continuous refractory period. But elevating pH increased excitability threshold during the normally superexcitable period even to a stronger extent as compared to the effect in acid pH.

Threshold responses to subthreshold conditioning stimuli are strongly affected by pH.

At the end of a 100 ms depolarizing conditioning stimulus the threshold reduction (i.e. the decrease in current required to elicit the target response) increased significantly with raising pH (pH 6.9: 40 ± 3 %; pH 7.4: 48 ± 3 %; pH 8.1: 52 ± 2 %; P<0.01, ANOVA) and was significantly lower in acid pH than in normal pH (P<0.01, paired t-test) but not different between normal and basic pH (P=0.17, paired t-test; Fig. 6A, B). The relation between the threshold reduction and the end of the depolarizing subthreshold stimulus during continuous changes

of the pH was best described with polynomial fit of the 2nd order (R^2=0.78, n=146, P<0.01; Fig. 6C). This function curve illustrates that low pH affects excitability alterations more than high pH when the nerve membrane is preconditioned with subthreshold depolarizing stimuli. In contrast to the application of a -40% hyperpolarizing conditioning stimulus of 100 ms excitability was more affected by basic pH than acid pH (pH 6.9: -164 ± 3 %; pH 7.4: -164 ± 22; pH 8.1: -117 ± 13 %; P<0.01, ANOVA; Fig. 7A, B). The threshold reduction (i.e. increase in threshold) was significantly less in basic pH compared to normal pH (P<0.05, paired t-test). No further threshold reduction was observed by lowering pH.

Applying a -120% conditioning stimulus for 300 ms led to less threshold current (decrease in threshold reduction) with increasing pH (pH 6.9: -342 ± 20 %, pH 7.4: -318 ± 15 %; pH 8.1: -277 ± 17 %; P<0.05, ANOVA; Fig. 7A, B). No difference in threshold was found between acid and normal pH (*P*=0.13, paired t-test). However, we found a significant difference between basic pH and normal pH (P<0.05, paired t-test). The relation between hyperpolarizing electrotonus and pH was best described with polynomial fit of the 2nd order (R^2=0.96, n=150, P<0.01; Fig. 7C).

The inward rectification in response to a hyperpolarizing stimulus results in a typical ‘sag’ towards control threshold starting with a latency of about 80 ms. The magnitude of this response is measured as the nadir of the threshold reduction (i.e. the peak current required to evoke the target response during hyperpolarization) and the plateau that is reached by 300 ms. This measurement is also referred to as S3 phase and is thought to reflect the effect of Ih-currents *(Burke et al., 2001)*. In our experiments, this inward rectification was significantly stronger in normal pH than in acid (P< 0.05, paired t-test) but not different to basic (P=0.18, paired t-test) pH (pH 6.9: 95 ± 15 %; pH 7.4: 157 ± 24 %; pH 8.1: 123 ± 18 %).

Discussion

This is the first study describing the effects of protons on nerve excitability parameters of myelinated sensory axons in vitro using threshold tracking. By using an in vitro preparation we can exclude many secondary factors that are precipitated by pH changes in vivo, including, changes of potassium levels which in turn affect nerve excitability.

Impact of pH on excitability is dependent on membrane potential.

The overall effect of pH on nerve excitability can be described as an increase of excitability threshold with decreasing pH. More current is needed to evoke the same size of the compound action potentials which implies that the resistance over the nerve membrane is increasing in parallel to the intra-neural concentrations of protons. Already 70 years ago, Lehmann suggested a strong linear relationship between pH and excitability threshold *(Lehmann, 1937)*. The technique of threshold tracking now suggests that the relationship of the control threshold to pH is not linear but rather described by a polynomial function of the third order. Furthermore, this technique also illustrates that the sensitivity of the neuronal membrane to excitability changes caused by pH depends strongly on the membrane potential. E.g. threshold changes caused by low pH are more pronounced when the membrane potential already is depolarized by subthreshold conditioning currents. On the other hand changing pH in the basic range affects excitability more when the membrane is hyperpolarized.

Persistent sodium conductances are modulated by pH.

The shortening of SDTC with simultaneously increasing of the rheobase at lower pH can be attributed to the blocking of persistent sodium currents. According to a study by Baker et al., pH has a great impact on those currents *(Baker and Bostock, 1999)*. In their investigation they showed that acid pH was

blocking late sodium currents by reducing the number of contributing channels while also reducing the single channel conductance. As a result, strength-duration time constant became shorter and the rheobase increased. Similarly, threshold tracking in human studies revealed that the fraction of persistent sodium currents on the node is an important contributor to differences in strength-duration time constant *(Bostock and Rothwell, 1997)*. Another investigation in human subjects by Mogyoros et al. *(1997)* could show a longer strength-duration time constant in volunteers during hyperventilation (e.g. elevating plasma pH) over minutes which was accounted to the function of persistent sodium channels. Our data are very much in accordance with those findings. Furthermore, the correlation of SDTC and pH in our investigation was not linear. The function curve suggests, that the blocking effect of pH on persistent sodium channels becomes more pronounced in basic pH.

Modulation of slow (GKs) and fast (GKf) potassium conductances by pH

The effect of pH on potassium currents was modest. However, a slowing of the kinetics of GKs in acid solutions could be shown in several ways. The excitability measure most directly related to GKs is the difference between recovery cycles following one and 7 conditioning stimuli, which is completely blocked by the KCNQ (Kv7) channel blocker XE991 (*Schwarz et al., 2006*). This is because activation of these slow channels, which cause the late subexcitability in the recovery cycle, accumulates during a brief train of action potentials, whereas superexcitability does not. Fig. 5C shows that this subexcitability difference is reduced in amplitude and prolonged in acid solution. Because of the slowed kinetics of inactivation, it is not clear whether the reduced amplitude of the threshold difference at short ISIs was due to a reduction in the maximum current or a reduced rate of activation during the conditioning action potentials. Further evidence for the effect of acidification on GKs is revealed by the changes in the accommodating ‘sag’ in depolarizing electrotonus (Fig. 6A), a

manifestation of KCNQ activity that is also abolished by XE991 (*Schwarz et al., 2006*). The accommodation is prolonged and apparently increased, while the post-depolarization undershoot in the threshold change is also increased and prolonged in acid solution. This might suggest that GKs is increased as well as slowed, but this seems unlikely in view of the observations of the effects of pH changes on KCNQ2 currents in vitro (*Prole et al., 2003*). The increased amplitude of undershoot, and corresponding overshoot in threshold change after hyperpolarization (Fig. 7A) more likely reflect block by protons of other channels contributing to the resting conductance, or due to a slight shift in resting membrane potential. Another complication in interpreting the changes in threshold electrotonus is that the block of KCNQ2 currents by protons is potential dependent (*Prole et al., 2003*).

In contrast to the changes in GKs, there is little evidence that the fast potassium conductance (GKf) is sensitive to pH. Recent studies of patients with episodic ataxia type 1, in which mutations in Kv1.1 channels reduce fast potassium channel function, have shown very prominent increases in superexcitability and also in peak excitability during depolarizing electrotonus with currents 20% of threshold (*Tomlinson et al., 2010*). No such changes were observed with the pH changes used in this study, although it has been reported that intra-axonal acidification can block fast K+ channels in rat spinal roots (*Moody Jr, 1984; Schneider et al., 1993*).

Inwardly rectifying currents (I_h) are not affected by pH.

Long hyperpolarizing conditioning currents lead to an activation of Ih (*Burke et al., 2001*). After the initial fanning out of the threshold the threshold started to pull back to a normal threshold showing the second component (S3, (*Bostock et al., 1998*)) which generally is attributed to activation of I_h. Using longer and more hyperpolarizing conditioning currents (400ms, 120%) however

illustrated even better the 'sagging in' of the S3 component. It also showed that the extent of this 'sagging in' was not statistically different in acid and basic, which means that rectification of inwardly rectifying currents, I_h, was comparable. The fanning out which could be seen during the first 100ms therefore more likely must be attributed solely to internodal potassium currents. The overshoot after the end of the conditioning is more pronounced in acid pH which reflects again the more extended block of rectifying slow potassium channels during hyperpolarization rather than the deactivation of I_h. If I_h was in fact more activated in basic pH, we would also expect a more extensive overshoot.

Clinical Implication

The clinical symptoms of hyperventilation-induced alkalosis on peripheral nerve (such as paresthesias, fasciculations or involuntary muscle contractions) have been attributed to hypocalcemia (*Kugelberg, 1948*; *Macefield, 1991*), since plasma proteins can reduce the ionised (and physiologically relevant) fraction of calcium in an alkaline milieu. In our *in-vitro* preparation, however, we also saw a clear increase in excitability as a reduction in pCO_2 produced an alkaline shift, but there were no plasma proteins to translate this into low ionised calcium. Our results therefore strongly support an alternative explanation, that the peripheral nerve hyperexcitability during hyperventilation results primarily from an increase of persistent sodium currents at basic pH (*Baker and Bostock, 1997*; *Mogyoros et al., 1997*).

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Table 1: Overview of excitability changes caused by pH

Excitability parameter	pH 6.7	pH 7.4	pH 8.1	pH 6.7 v 8.1
Stimulus-response				
Peak response (mV)	3.41 ± 0.16	3.23 ± 0.09	3.48 ± 0.24	
Stimulus for 50% max. response (mA)	**0.61 ± 0.10***	0.51 ± 0.08	**0.39 ± 0.06****	**
Stimulus-response slope	2.30 ± 0.26	2.70 ± 0.32	**1.99 ± 0.18****	
Strength-duration relationship				
Strength-duration time constant (ms)	0.20 ± 0.04	0.20 ± 0.03	**0.31 ± 0.05***	**
Rheobase (mA)	0.43 ± 0.07	0.32 ± 0.04	**0.24 ± 0.04****	***
Recovery cycle				
Relative refractory period (ms)	2.7 ± 0.5	1.9 ± 0.2	2.3 ± 0.5	
Peak superexcitability (%)	-14.7 ± 2.4	-15.8 ± 2.1	-13.3 ± 2.4	
Peak subexcitability (%) (1 cond. stim.)	7.2 ± 1.6	5.6 ± 0.5	6.7 ± 1.1	
Extra subex.(5-10ms)(7-1 cond. stims.)	**25.4 ± 1.7*****	42.3 ± 1.9	48.4 ± 4.9	***
Extra subex.(50-80ms)(7-1 cond. stims.)	**8.4 ± 0.8***	4.5 ± 0.8	**2.0 ± 0.6***	***
Threshold electrotonus				
TEd^{40}(peak)(%)	**55.2 ± 3.8****	61.6 ±2.7	59.6 ± 2.4	
TEd^{40}(90-100ms)(%)	**40.0 ± 3.2***	48.2 ± 1.5	52.1 ± 2.4	**
TEd^{40}(S2 accommodation)(%)	15.2 ± 2.1	13.4 ± 1.4	**7.5 ± 1.3****	**
TEd^{40}(undershoot)(%)	-17.5 ± 2.1	-16.2 ± 1.2	**-10.2 ± 2.5***	
TEh^{40}(overshoot)(%)	**19.2 ± 2.6***	13.7 ± 2.5	10.5 ± 1.1	**
TEh^{40}(90-100ms)(%)	-164 ± 22	-164 ±22	**-117 ± 12.6***	*
Negative peak				
Latency (ms)	1.59 ± 0.06	1.57 ± 0.04	1.57 ± 0.04	

Table Legend

Values are mean ± SE. Overview of excitability changes related to pH. Comparison of nerve excitability parameters between recordings at 3 different pH. Asterisks in columns for pH 6.7 and 8.1 indicate significant differences from recordings at pH 7.4 (paired t-tests), last column indicates significant differences between pH 6.7 and 8.1 (paired t-tests): * = P < 0.05, ** = P < 0.01, *** = P < 0.001.

Figure Legends

Figure 1. Schematic representation of the preparation.

The skin nerve in vitro preparation was superfused with carbogen-gassed SIF maintaining the pH at 7.40 and 32° C throughout the experiment. A pair of self-sealing stainless steel rings isolated a short stretch of nerve. The flow of the inner compartment (inner diameter 8 mm) was separately controlled and pH could be changed by altering the flow of CO_2.

Figure 2. Representative illustration of pH and threshold changes

A: Specimen of the time course of the control threshold (current required to elicit a 40% of the maximal SNAP amplitude, upper trace) and pH (lower trace). After reaching steady state the first comprehensive nerve excitability test (NET) was carried out at a pH of 7.40. By reducing the CO_2 flow the pH then increased over 20 minutes until a plateau was reached at a pH of ~8.15. Note the delay of 6 minutes that is caused by the delay circulation from the reservoir. A second NET was carried out at basic pH and subsequently the pH was lowered to pH ~6.8 by slowly increasing the CO_2 concentration over 40 minutes until a plateau was reached and the third NET reading was taken.

Figure 3. Reduced nerve excitability threshold in basic pH

A: The stimulus-response curve is shifted towards higher current values in low pH compared to high pH. B: Accordingly, the current required for the 50% of the maximal SNAP amplitude was significantly higher in low pH (0.61 ± 0.10 mA) than in normal pH (0.51 ± 0.08 mA) or high pH (0.39 ± 0.06 mA), (P<0.01, ANOVA). C: mean ± SEM of the control threshold changes normalized to the threshold at a pH of 7.40 (dotted lines). The curve was best fitted with a 3rd order polynomial fit (R^2=0.99, P<0.01).

Figure 4. Strength duration time constant and rheobase are modulated by pH

A. the strength-duration time constant (SDTC) is represented by the negative intercept of the fitted line with the x-axis. It was significantly longer (B) in basic pH (0.31 ± 0.05 ms) than in normal (0.20 ± 0.03 ms, $P<0.05$, paired t-test) or acid pH (0.20 ± 0.03 ms, $P<0.01$, paired t-test). Rheobase, represented by the slope of the fitted line, is higher in acid pH (0.40 ± 1.2 mA) compared to normal (0.30 ± 1.15 mA, $p<0.01$, paired t-test) or basic pH (0.22 ± 1.2 mA, $P<0.001$, paired t-test). The mean changes in SDTC are best fitted with a 2nd order polynomial fit ($R^2=0.99$, $P<0.01$).

Figure 5. The recovery cycle is affected by pH change

A, Mean ± SEM of the recovery cycles following 1 supramaximal conditioning stimuli. The relative refractory period and the subexcitability after a single conditioning stimulus did not differ significantly at acid (RRP: 2.5 ± 1.2ms, Subex.: 7 ± 2 %,), neutral (RRP: 1.9 ± 1.2ms; Subex.: 6 ± 1%) or basic (RRP: 2.1 ± 1.2ms; Subex.: 7 ± 1 %) pH. B. There were significant changes of the late subexcitability after 7 conditioning stimuli The nadir between 1ms and 10ms delay was significantly lower in acid (11 ± 4 %) than in basic (41 ± 8 %, $P<0.01$, paired t-test) but not different compared to normal (21 ± 8 %) pH. C. Recovery cycles showing the differences between 7 and 1 conditioning stimuli. The difference can be attributed almost solely to the Ks current.

Figure 6. Depolarizing threshold electrotonus (TEd) is influenced by pH

A. mean ± SEM of threshold changes in response to a 40%, 100 ms-conditioning subthreshold current (threshold electrotonus) plotted as threshold reduction. B,

Threshold reduction (i.e. requirement of less current) was significantly stronger in basic pH than in acid pH (P<0.01, ANOVA). At the end of the 100 ms depolarizing conditioning stimulus the threshold reduction was significantly lower (i.e. requirement of more current) in acid (40 ± 3 %) than in neutral (48 ± 3 %, p<0.05, paired t-test) or basic pH (52 ± 2 %, p<0.01, paired t-test). Accordingly, the undershoot of the threshold reduction after the end of the depolarizing conditioning stimulus was more pronounced in acid than in basic (*P*=0.01, ANOVA) pH. The relation between the threshold reduction in response to the depolarizing conditioning stimulus at 100 ms and the pH was best described with polynomial fit of 2nd order (R^2=0.97, P<0.01).

Figure 7. The hyperpolarizing threshold electrotonus is influenced by pH changes.

A, mean ± SEM of the threshold reduction after a subthreshold hyperpolarizing conditioning stimulus of -40% for 100 ms (left panel) and of -120% for 300 ms (right panel). B, at the end of 100 ms, the negative threshold reduction (i.e. increase in threshold) was less pronounced in basic (-117 ± 13 %) than in neutral (-164 ± 22 %, *P*<0.05, paired t-test) or acid pH (-164 ± 3 %, *P*<0.05, paired t-test). The slope of the recovery after the end of the 100 ms hyperpolarizing conditioning stimulus (101-140 ms, i.e. 1 to 40 ms after the end of the conditioning stimulus) was significantly steeper in basic than in acid pH (pH 6.9: 2.76 ± 0.46 %, pH 8.1: -1.86 ± 0.30 %, *P*<0.01). After a -120% conditioning stimulus the difference in threshold reduction at 300 ms between acid and basic pH was even more distinct (pH 6.9: -342 ± 20 %, pH 8.1: -277 ± 17 %, *P*<0.01). The relationship between threshold reduction in response to a -40% conditioning stimulus lasting 100ms and pH was best described with polynomial fit of the 2nd order (R^2=0.96, n=150, *P*<0.01). The rectification, measured as the difference of threshold reduction between the nadir of the threshold reduction and the sag measured at a delay of 300 ms (S3 phase, *(Burke et al., 2001)*) was not

significantly different between acid and basic pH (pH 6.9: 95 ± 15 %, pH 8.1: 123 ± 18 %).

Figure 1

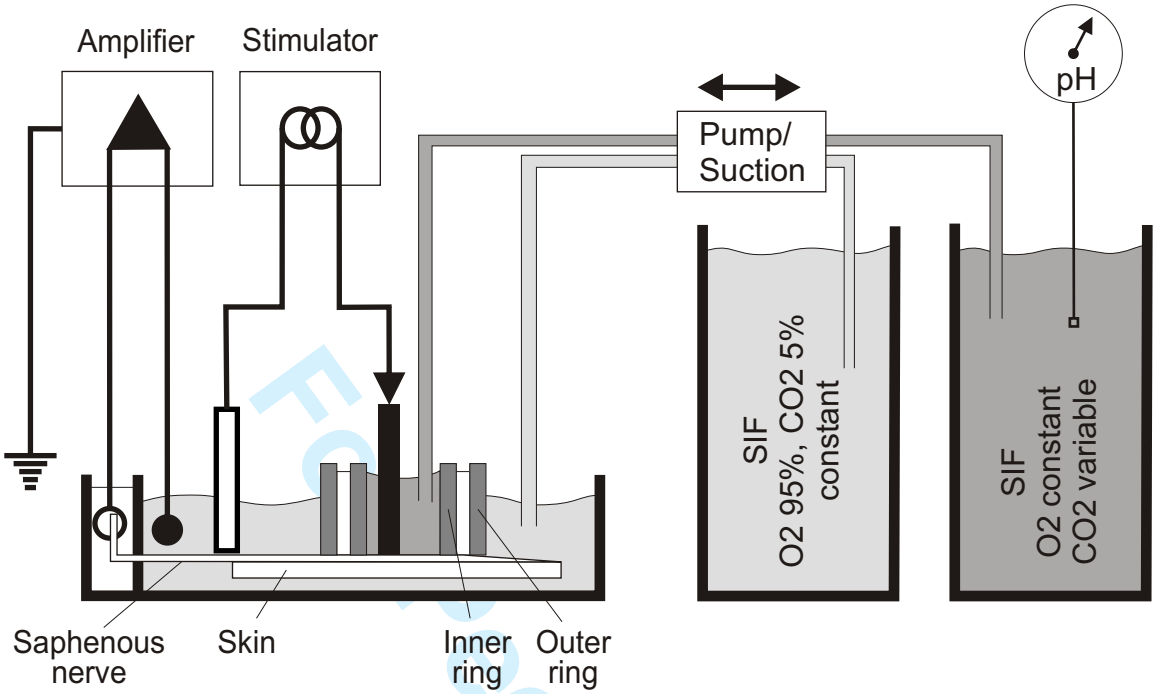


Figure 2

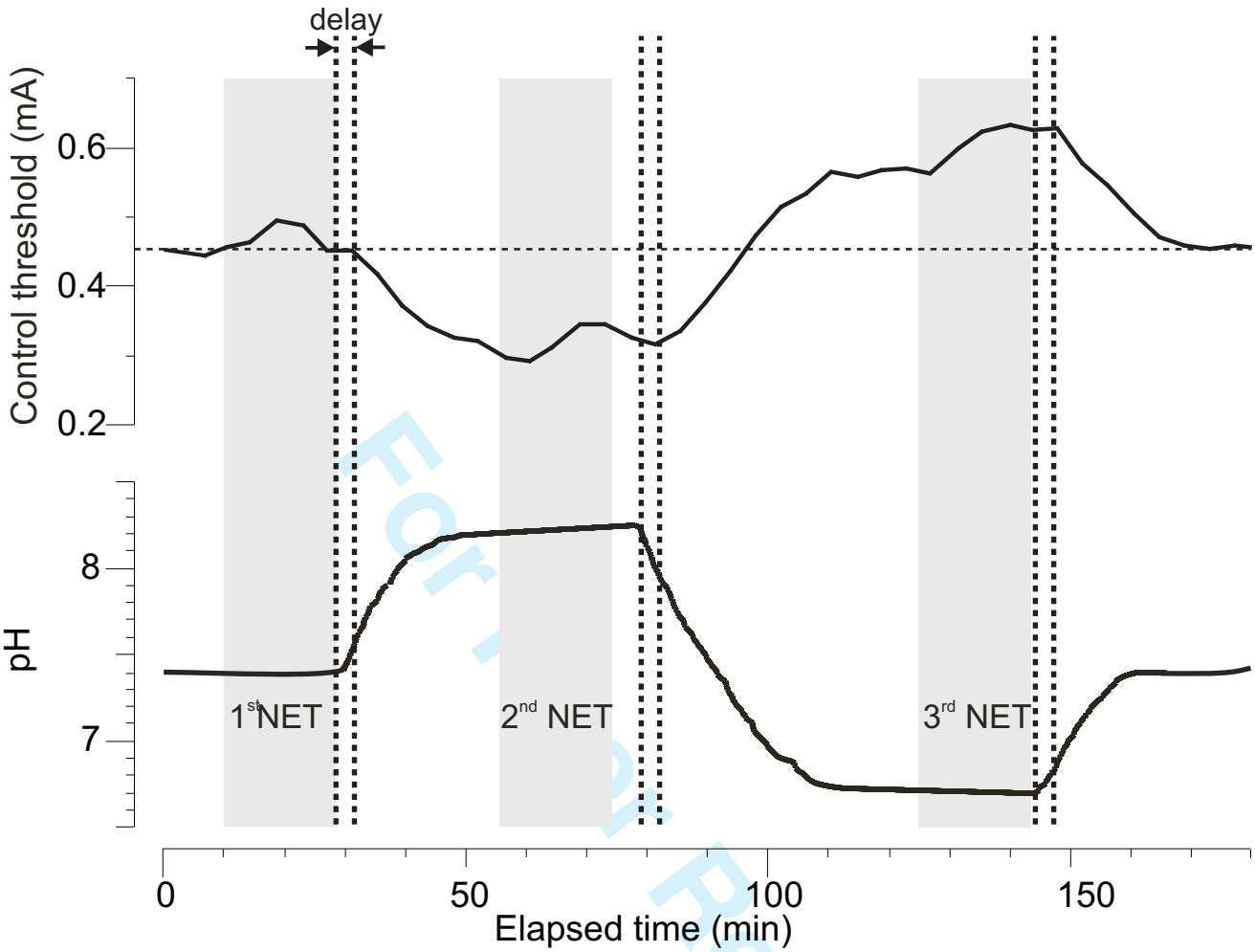


Figure 3

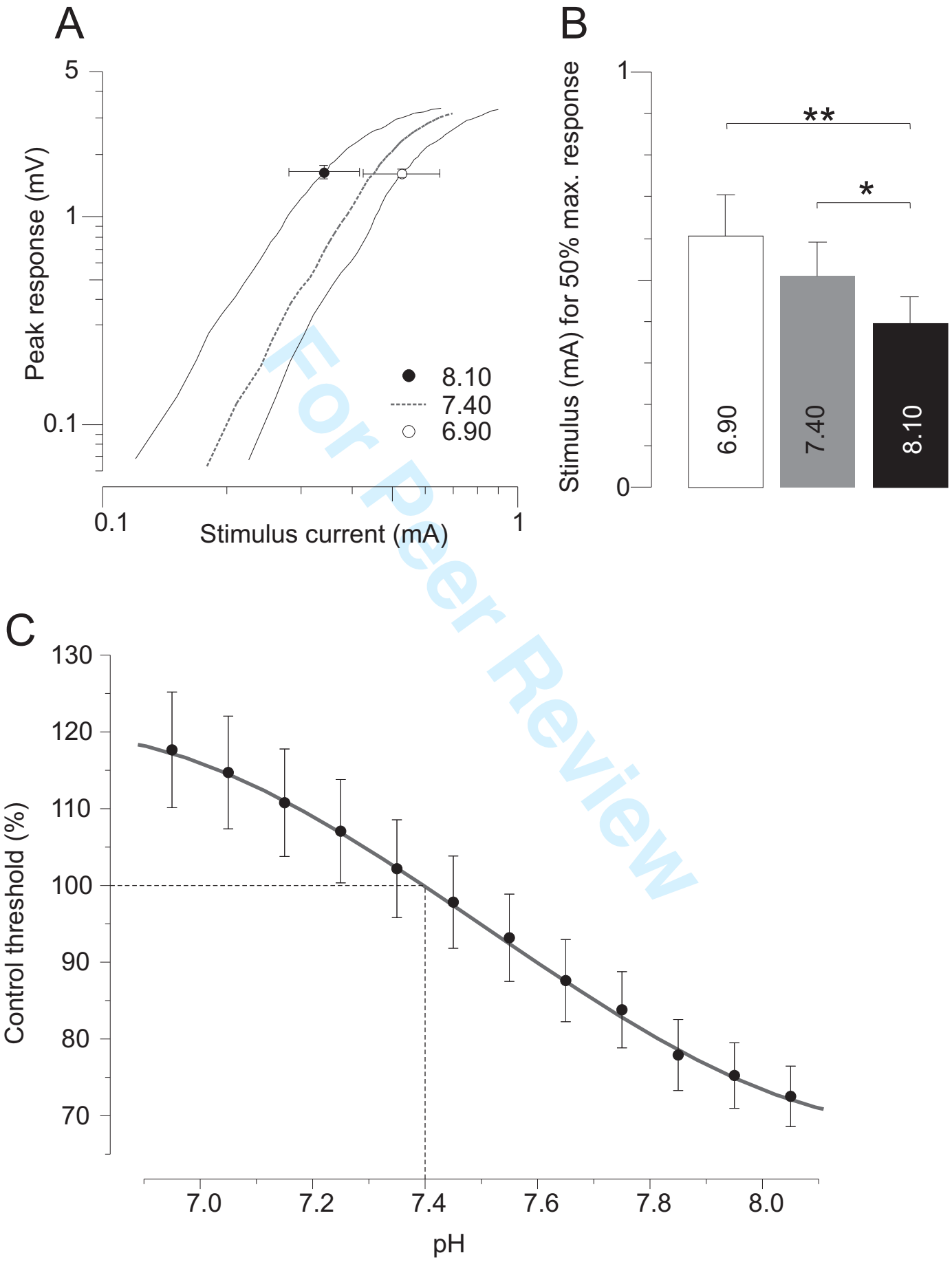
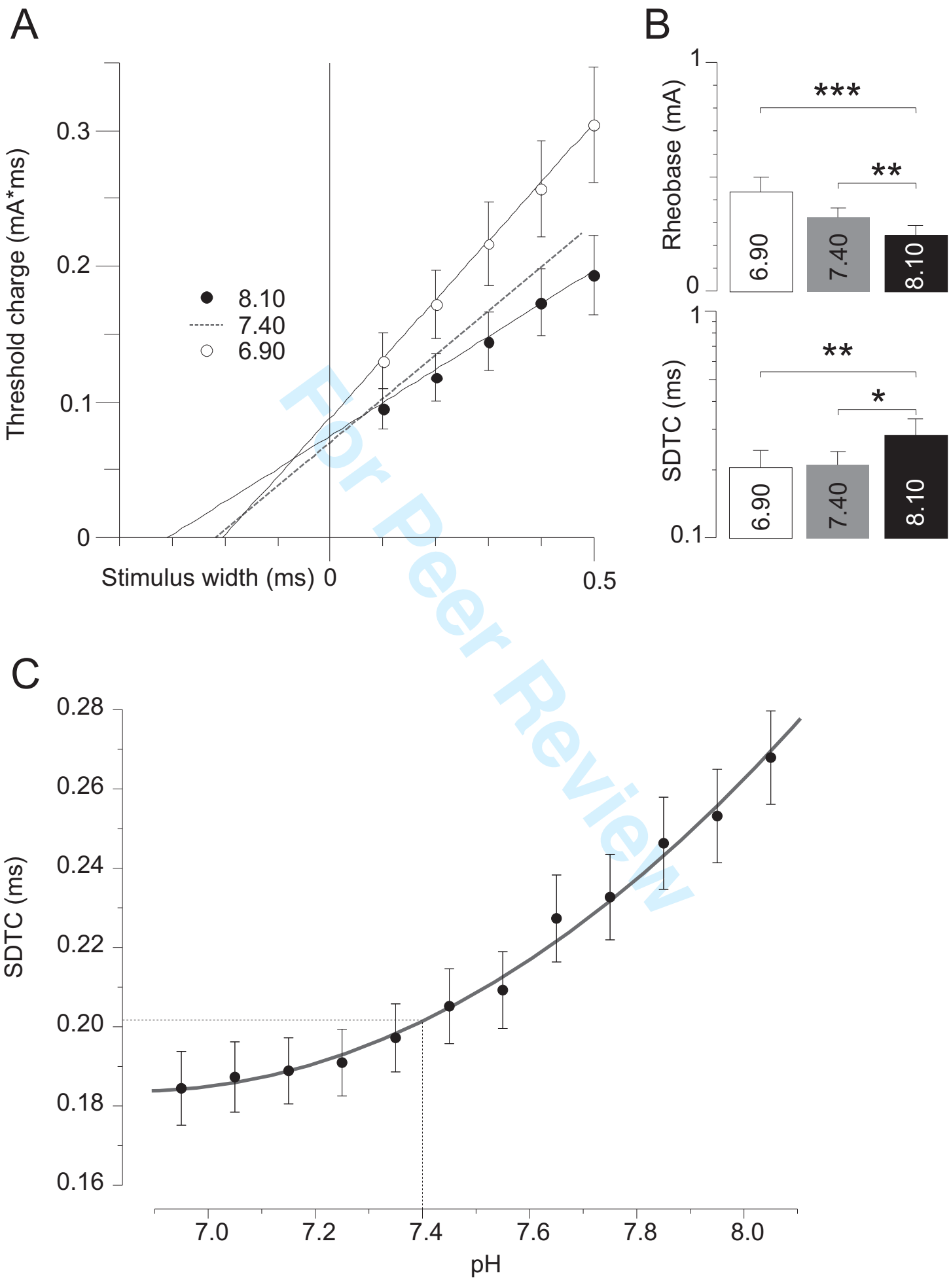


Figure 4



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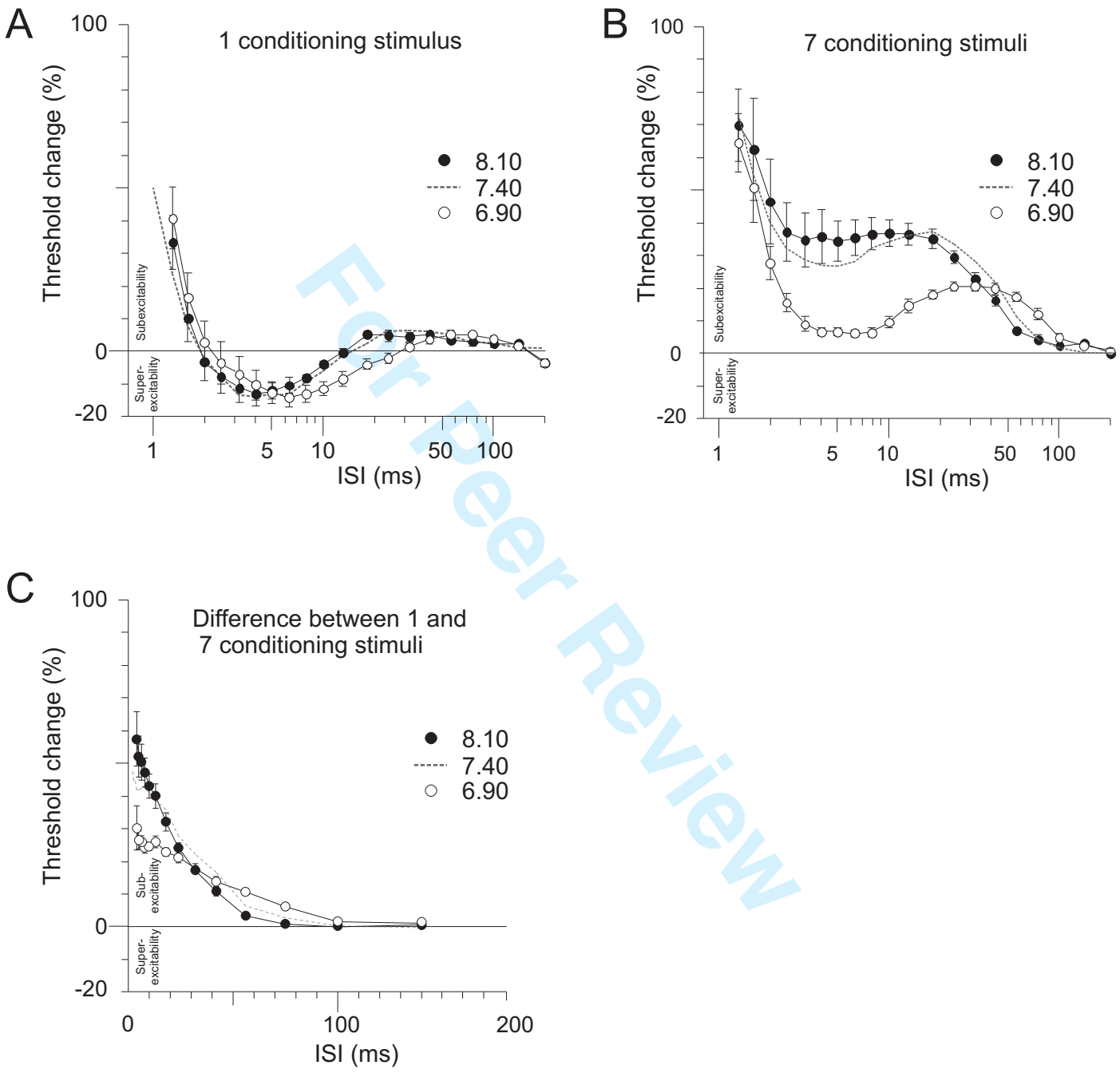

Figure 6

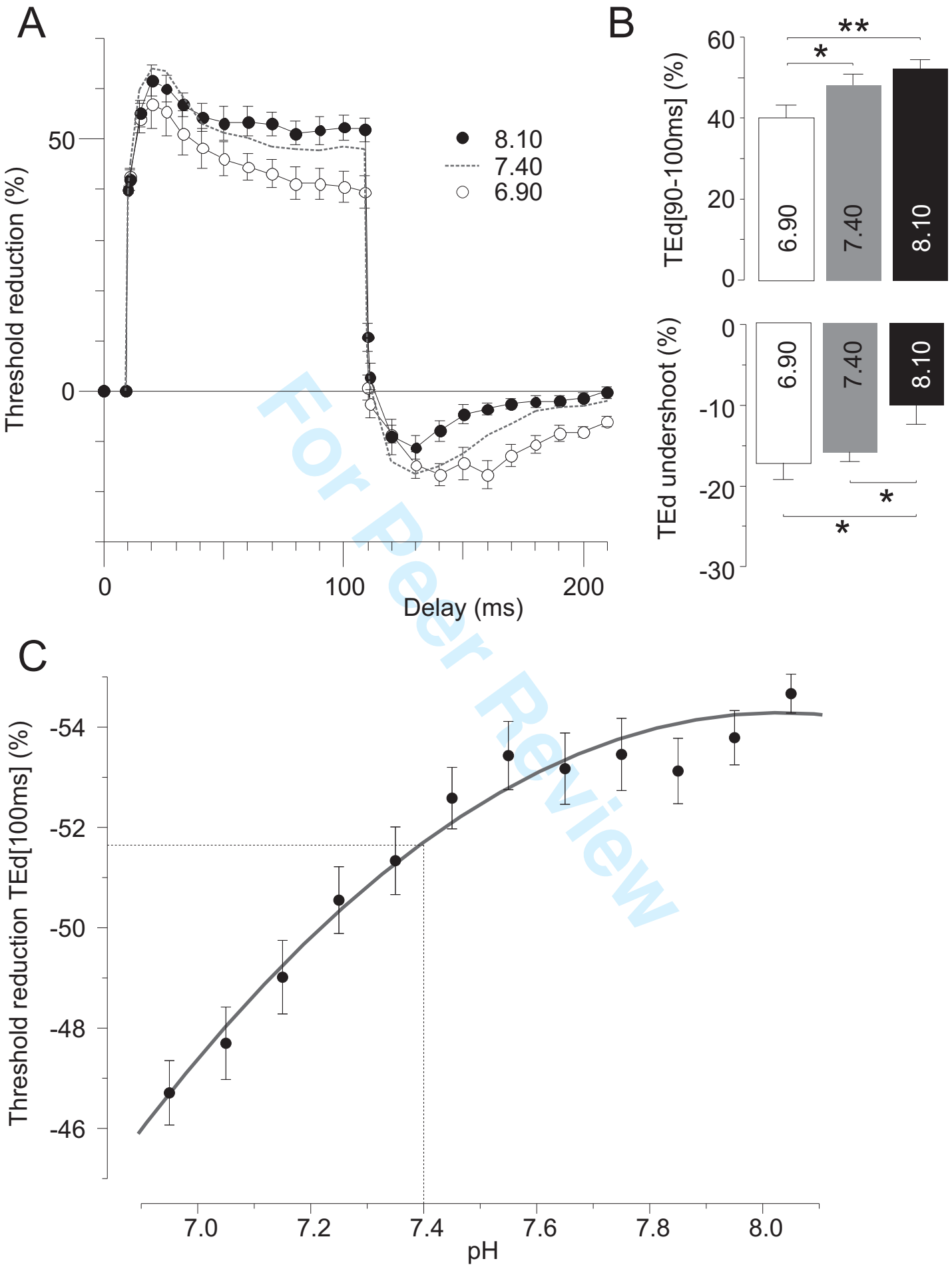

Figure 7